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Cytotherapy 26 (2024) 1105–1117

Contents lists available at ScienceDirect

CYTOTHERAPY

journal homepage: www.isct-cytotherapy.org

FULL-LENGTH ARTICLE
Manufacturing

Validating human induced pluripotent stem cell-specific quality control tests for the release of an intermediate drug product in a Good Manufacturing Practice quality system

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ARTICLE INFO

Article History:
Received 25 January 2024
Accepted 10 April 2024

Key Words:
GMP
hiPSC
quality control
validation

ABSTRACT

One of the challenges in Good Manufacturing Practice (GMP)-compliant human induced pluripotent stem cell (hiPSC) production is the validation of quality control (QC) tests specific for hiPSCs, which are required for GMP batch release. This study presents a comprehensive description of the validation process for hiPSC-specific GMP-compliant QC assays; more specifically, the validation of assays to assess the potential presence of residual episomal vectors (REVs), the expression of markers of the undifferentiated state and the directed differentiation potential of hiPSCs. Critical aspects and specific acceptance criteria were formulated in a validation plan prior to assay validation. Assay specificity, sensitivity and reproducibility were tested, and the equipment used for each assay was subjected to performance qualification. A minimum input of 20 000 cells (120 ng of genomic DNA) was defined for accurate determination of the presence of REVs. Furthermore, since vector loss in hiPSC lines is a passage-dependent process, we advocate screening for REVs between passages eight and 10, as testing at earlier passages might lead to unnecessary rejection of hiPSC lines. The cutoff value for assessment of markers of the undifferentiated state was set to the expression of at least three individual markers on at least 75% of the cells. When multi-color flow cytometry panels are used, a fluorescence minus one control is advised to ensure the control for fluorescent spread. For the assay to assess the directed differentiation potential, the detection limit was set to two of three positive lineage-specific markers for each of the three individual germ layers. All of our assays proved to be reproducible and specific. Our data demonstrate that our implemented analytical procedures are suitable as QC assays for the batch release of GMP-compliant hiPSCs.

Introduction

Human induced pluripotent stem cells (hiPSCs) are a promising cell source for regenerative cell therapy because of their ability to differentiate into various cell types. Production of hiPSC-derived cell therapies requires the use of Good Manufacturing Practice (GMP)-compliant hiPSCs as starting material. One of the challenges in GMP-compliant hiPSC production is addressing the lack of qualified and validated hiPSC-specific quality control (QC) tests required by the qualified person for release in a GMP quality system. According to current GMP guidelines, validation of analytical procedures is defined as "establishing documented evidence of data demonstrating that the proposed testing and acceptance criteria are sufficiently under control to guarantee reproducible quality of the products at release and adequate control during shelf-life (stability)" [1,2]. QC tests used frequently for cell and gene therapy products are established, their acceptance criteria are well defined and they are performed according to the guidelines of the European Pharmacopoeia (e.g., mycoplasma,

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https://doi.org/10.1016/j.jcyt.2024.04.004

sterility and endotoxin) [3–6]. The situation is different for hiPSC-specific QC tests, as their qualification and validation, including the establishment of acceptance criteria for their use in a GMP-compliant production process, are not well described in the current literature.

For intermediate drug product release of our in-house manufactured GMP-compliant hiPSCs [7], we qualified and validated three hiPSC-specific release tests. Acceptance criteria were based on recommendations from the Dutch Bureau for Genetically Modified Organisms, the International Society for Stem Cell Research Standards for Human Stem Cell Use in Research [8] and recommendations described previously [9,10]. Assay design was such that it adhered to European Union guideline part IV regarding GMP for advanced therapy medicinal products [11] and was in line with ICH harmonized guidelines Q2 (validation of analytical procedures) and Q14 (analytical procedure development) [12], taking into account that this is an intermediate drug product that will be used in early clinical development. Although our choice for the following described methods was based on the aforementioned guidelines and recommendations, it was also influenced by current practice in our research setting. The use of existing analytical methods routinely applied in one's own institute facilitates development and simplifies risk management. The hiPSC-specific release test we chose to validate was residual episomal vector (REV) analysis (our production process makes use of episomal vectors) [13]. Although described as an "integration-free" method, vector loss is a passage-dependent process, and in some cases, integration in genomic DNA (gDNA) may occur [14]. This underscores the need to implement assays that identify REV retention, especially for hiPSCs intended for clinical use. With regard to markers of the undifferentiated state, well-known markers include transcription factors organic cation transporter 3/4 (OCT3/4), sex determaning region Y box transcription factor 2 (SOX2) and Homeobox protein NANOG (NANOG) as well as tumor rejection antigens TRA-1-60 and TRA-1-81 and stage-specific embryonic antigens (SSEA)3 and SSEA4 [15,16]. The use of markers to establish that input material for cell therapy product manufacture is in the undifferentiated state and, as much as possible, devoid of already differentiated cells is an important aspect of QC analysis. With respect to functional pluripotency, this can be determined by evaluating the potential of hiPSCs to differentiate into progenitors of definitive endoderm, mesoderm and ectoderm [8]. Functional pluripotency is of crucial importance, as GMP-compliant lines should have the potential to differentiate into a vast number of different cell products for clinical use. The ability to produce multiple putative clinical products from the same master cell bank (MCB) is cost-effective.

Although hiPSC-specific tests are used by research labs to determine quality of research-grade hiPSCs and proposed acceptance criteria can be found in the literature [10,17], it is essential to validate them for GMP-compliant production processes. This study presents a comprehensive description of the validation process for hiPSC-specific GMP-compliant QC assays and proposes test-specific acceptance criteria.

Methods

Generation of hiPSC lines

Peripheral blood samples were collected from healthy male donors aged 18–30 who provided their consent for the utilization of their donated material for research-grade hiPSC generation. In addition, we collected peripheral blood from healthy male donors who granted their consent for the use of their donated material for GMP hiPSC production purposes and use in clinical research, commercialization and whole genome sequencing (WGS). Male donors were chosen to avoid possible erosion of X chromosome inactivation described for hiPSC lines from female donors [18]. Whole blood was used for the isolation of peripheral blood mononuclear cells (PBMCs) using a density gradient separation procedure and further enriched for erythroblasts with StemSpan-ACF medium (09860; STEMCELL Technologies, Vancouver, Canada) supplemented with StemSpan Erythroid Expansion Supplement 100× (02692; STEMCELL Technologies). Erythroblasts were transfected with episomal vectors described by Okita *et al.* [13]—pCXLE-hOCT3/4 (27076; Addgene, Watertown, MA, USA), pCXLE-hSK (27078; Addgene) and pCXLE-hUL (27080; Addgene)—using $0.5–1 \times 10^6$ live cells. Cell pellets were resuspended in P3 buffer (P3 Primary Cell 4D-Nucleofector X Kit, V4XP-3012; Lonza, Basel, Switzerland) containing 1 μg of each episomal vector. Electroporation was performed in a Nucleocuvette Vessel (Lonza) using the 4D-Nucleofector (Lonza) program EO-115. After reprogramming, hiPSC colonies were selected and expanded as independent clonal lines using mTeSR Plus medium (100-0276; STEMCELL Technologies) on plates coated with Biolaminin 521 CTG 0.5 μg/cm^2 (CT521; BioLamina, Sundbyberg, Sweden) until passage two. After passage two, the hiPSCs were maintained on plates coated with Vitronectin 0.9 μg/cm^2 (A27940; Thermo Fisher Scientific, Waltham, MA, USA). All lines were coded using donor number, passage number and line number. Cells were harvested at different passages using CTS Versene (A4239101; Thermo Fisher Scientific) at 37°C for 10 min to produce single cells or at room temperature for 4 min to produce cell aggregates [7].

Generation of an EBNA-1*-positive control*

A positive control line with stably integrated *EBNA-1* was generated using the STRAIGHT-IN acceptor hiPSC line LU99_CLYBL-bxb-v2 [19]. The Bxb1 donor plasmid containing *EBNA-1* was assembled by inserting a polymerase chain reaction (PCR)-amplified fragment of the *EBNA-1* sequence into the plasmid pBR attB(bxb) LOX (183762; Addgene) linearized by the restriction enzyme NheI (R3131S; New England Biolabs, Ipswich, MA, USA) using the NEBuilder HiFi DNA Assembly Cloning Kit (E5520S; New England Biolabs). The primers used to amplify the *EBNA-1* fragment from the pCXLE-hOCT3/4 plasmid were EBNA1_HiFi-F (5′-CGGCAAGCAGGCATCGACTAGTTAATTAAGCATCGTGGTCAAGGAGGTT-3′) and EBNA1_HiFi-R (5′-AAGTTATCGTGAGGAAGAGTTCTTGCAGCGTGTCATAACAAGGTCCTTAATCGCATCC-3′). The donor plasmid was then integrated into the acceptor hiPSC line LU99_CLYBL-bxb-v2 to generate the *EBNA-1*-positive control line LU99_EBNA-1. The presence of a single *EBNA-1* copy in the gDNA was confirmed with droplet digital PCR (ddPCR) (see supplementary Figure 1).

Culture of HaCaT cells

Human immortalized keratinocyte (HaCaT) cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (11965092; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (SH30071.03; Cytiva, Marlborough, MA, USA) in a six-well cell culture plate. Cells were harvested using TrypLE Select 1× (12563011; Thermo Fisher Scientific) at 37°C for 10 min and washed with Dulbecco's Modified Eagle's Medium/F12 before counting.

Cell count and viability

Cell numbers and viability were assessed using a NucleoCounter NC-200 (ChemoMetec, Lillerød, Denmark) according to the manufacturer's recommendations.

DNA isolation

The gDNA was extracted from a maximum of 5×10^6 PBMCs and hiPSCs at indicated passage numbers using the DNeasy Blood & Tissue Kit (69504; QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. The DNA concentration was measured in triplicate using a NanoDrop ND-1000 spectrophotometer (Thermo

Fisher Scientific) according to the manufacturer's recommendations. DNA samples were diluted to a final concentration of 60 ng/μL.

Quantitative PCR

The quantitative PCR (qPCR) reaction was prepared in triplicate using PowerTrack SYBR Green Master Mix (A46012; Thermo Fisher Scientific) according to the manufacturer's recommendations with gDNA concentrations ranging from 120 ng to 12 pg (corresponding to 20 000 to two copies of *EBNA-1*), 250 nM forward primer and 250 nM reverse primer in a total volume of 10 μL. Primers for the detection of *EBNA-1*, which is present in the backbone of each reprogramming vector, were EBNA-F-primer (5′-CAAGGAGGTTCCAACCCGAA-′3) and EBNA-R-primer (5′-GACCCAAGTTCCTTCGTCGG-′3), and primers for detection of the housekeeping gene hexokinase 2 (*HK2*) were HK2-F-primer (5′- GCCGACTCTTGTATTGCCTG-′3) and HK2-R-primer (5′-TATTGTAGCACGGCCGGAAA-′3). A positive control standard curve from the gDNA of LU99_EBNA-1 was used in every single run. Every qPCR plate also contained a negative control (LU99_CLYBL-bxb-v2) and a no template control (water). The qPCR was performed using the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific) with an initial denaturation step at 95°C for 3 min followed by a denaturation step at 95°C for 15 s and an annealing and extension step at 60°C for 45 s for 35 cycles. To asses specificity of the qPCR run melting curves were generated after 35 cycles by a denaturation step at 95°C for 1 min followed a slow melting step from 65°C to 95°C at increments of 0.5°C/s and a fluorescence read every second. To simplify visual interpretation Cq values were normalized by subtracting the obtained Cq value from the maximum number of cycles (35) This sets the undetermined values (no PCR product obtained) as 0.

Droplet digital PCR

We performed and analyzed ddPCR using a thermocycler (QX200 AutoDG) and the QX200 Droplet Digital PCR System; (Bio-Rad Laboratories, Hercules, CA, USA) with QuantaSoft software (Bio-Rad Laboratories). Assays comprising pre-mixes of forward and reverse primers (18 μM each) with a FAM-conjugated hydrolysis probe (5 μM) were designed for *EBNA-1*. Reactions (final volume 22 μL) were prepared with 2× ddPCR Supermix for Probes (no dUTP) (1863024; Bio-Rad Laboratories), 900 nM of each primer and 250 nM of each probe. Approximately 100 ng of gDNA digested with 2–5 U of HindIII-HF (R3104S; New England Biolabs) was added. Droplet generation, PCR amplification and analysis were all performed according to the manufacturer's instructions. An assay for the detection of *AmpR* was included as a positive control. The two-copy autosomal gene *RPP30* was used as a reference. The primers and probes used in this study are listed in Table 1.

Preparation of samples for Epstein–Barr virus infection analysis

To exclude the presence of Epstein–Barr virus (EBV) viral DNA in hiPSCs other than that of the EBNA-1 gene present in the reprogramming vectors, hiPSC samples were handed to the accredited routine test laboratory of the Leiden University Medical Center (LUMC) Department of Medical Microbiology to be subjected to EBV infection testing. The hiPSC samples cryopreserved at passage four were taken from liquid nitrogen and transported to the test lab. The routine test comprised TaqMan qPCR using the *BNRF1* gene as the EBV target with the primers 144EBV-F (5′-GGAACCTGGT-CATCCTTTGC-3′) and 145EBVR (5′-ACGTGCATGGACCGGTTAAT-3′) and TaqMan probe 510EBV-TQ-FAM (FAM 5′-CGCAGGCACTCG-TACTGCTCGCT-3′ TAMRA).

WGS and analysis

To assess possible integration of reprogramming vectors, hiPSCs at passage 10 were subjected to WGS, which was outsourced to the accredited company GenomeScan (Leiden, the Netherlands). WGS with a minimum sequence coverage of 50× was performed using the NovaSeq 6000 platform (Illumina, San Diego, CA, USA). The WGS analysis workflow entailed QC and a read pre-processing stage in which adapter sequences, low-quality regions (Phred <20) and poly-G trimming were performed. Reads shorter than 20 bp were discarded. Subsequently, reads were mapped with a short read aligner to the indexed reference sequence (GRCh37/hg19). The resulting alignment (BAM) files were sorted on coordinate and indexed for downstream analysis. For the transgenic sequence, possible integration into the host genome was checked using TC-hunter [21]. TC-hunter extracts alignment information contained in the sample BAM file, including discordant read pairs (those in which one read is aligned to the host and the other read is aligned to the construct) and chimeric reads (those in which a single read aligns to both the host and the construct). This information is used to detect the break point location(s) of the transgenic insertion region(s). The sample BAM file for this analysis was generated using BWA-MEM (v0.7.17) by aligning the adapter- and quality-trimmed reads to the reference sequence containing both the host genome sequence and the construct sequence.

Analysis of differentiation capacity

The STEMdiff Trilineage Differentiation Kit (05230; STEMCELL Technologies) was used for tri-germ layer differentiation analysis. Dissociated hiPSCs were plated on 13-mm coverslips coated with Matrigel hESC-Qualified Matrix (CLS354277; Corning, Corning, NY, USA) in 24 wells in mTeSR Plus medium supplemented with 10 μL/mL RevitaCell (Thermo Fisher Scientific) 1 day before differentiation induction to endoderm (4×10^5 cells) or mesoderm (1×10^5 cells). For ectoderm differentiation, 4×10^5 hiPSCs were plated in STEMdiff ectoderm medium (STEMCELL Technologies) supplemented with 10 μL/mL RevitaCell. Lineage-specific medium was changed daily. After 5 days for endoderm and mesoderm differentiation and 7 days for ectoderm differentiation, cells were fixed with 2% paraformaldehyde (16005; Sigma-Aldrich, St Louis, MO, USA) for 30 min at room temperature.

Table 1
Primer/probe assays used for ddPCR.

Target gene	Primer/probe	Sequence (5′–3′)	Fluorophore quencher	Source
EBNA	Forward primer	TACCGACGAAGGAACTTGGG	–	Present study
	Reverse primer	GCAGTTCCTCGCCTTAGGTT	–	
	Probe	CGGTGTGTTCGTATATGGAGGTAGTA	FAM-ZEN-IBFQ	
AmpR	Forward primer	TTTCCGTGTCGCCCTTATTCC	–	Roberts *et al.* [20]
	Reverse primer	ATGTAACCCACTCGTGCACCC	–	
	Probe	TGCCTTCCTGTTTTTGCTCACCCA	FAM-ZEN-IBFQ	
RPP30	Forward primer	GATTTGGACCTGCGAGCG	–	Present study
	Reverse primer	GCGGCTGTCTCCACAAGT	–	
	Probe	CTGACCTGAAGGCTCT	HEX-ZEN-IBFQ	

The differentiation capacity of the hiPSCs was confirmed by immunostaining using lineage-specific markers. The choice of markers was based on the marker set routinely and successfully used by our hiPSC hotel. A large batch was used to circumvent having to use different batches during the validation process. Fixed coverslips were washed twice with phosphate-buffered saline (PBS) before incubation with Triton blocking solution comprising 4% normal swine serum (014-000-121; Jackson ImmunoResearch, West Grove, PA, USA) in 0.1% Triton X-100 (93443; Sigma-Aldrich) for 1 h at room temperature. Coverslips were washed with PBS and incubated overnight at 4°C with custom-made pre-labeled antibodies from Cell Signaling Technology (Danvers, MA, USA) in 4% normal swine serum/PBS with 1:1000 4′,6-diamidino-2-phenylindole (62248; Thermo Fisher Scientific) as follows: for ectoderm, FAPB7 (D8N3N) at 1:100 (Alexa Fluor 555), PAX6 (D3A9V) at 1:200 (Alexa Fluor 647) and Nestin (10C2) at 1:200 (Alexa Fluor 488); for endoderm, FOXA2 (D56D6) at 1:500 (Alexa Fluor 555), GATA4 (D3A3M) at 1:200 (Alexa Fluor 647) and EOMES (D8D1R) at 1:100 (Alexa Fluor 488); for mesoderm, CDX2 (D11D10) at 1:500 (Alexa Fluor 555), vimentin (D21H3) at 1:400 (Alexa Fluor 647) and Brachyury (D2Z3J) at 1:200 (Alexa Fluor 488). Before mounting the coverslips with Mowiol (475904; Merck, Darmstadt, Germany), they were washed three times with 0.05% Tween 20 (822184; Merck) for 10 min in the dark.

For endoderm and mesoderm, images were taken in a single plane for each of the channels to obtain an overlay of all markers used. To acquire ectodermal three-dimensional structures, maximal projection was obtained by overlaying images taken in several z-planes. Images were produced at ×20 or ×63 magnification, or both, with an LSM 900 Airyscan confocal microscope using ZEN microscopy software (Zeiss, Oberkochen, Germany) or at ×40 magnification with an SP8 confocal microscope using LAS X software (Leica, Wetzlar, Germany).

Analysis of markers of the undifferentiated state

Dissociated single hiPSCs or HaCaT cells (negative control) were fixed and permeabilized using the BD Cytofix/Cytoperm Fixation/Permeabilization Kit (554714; BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's recommendations. Permeabilized cells (2×10^5 cells per fluorescence-activated cell sorting [FACS] tube) were incubated at 4°C for 30 min with fluorescently labeled antibodies. Cells were washed with BD Perm/Wash buffer (BD Biosciences) and FACS buffer (PBS/0.5% ethylenediaminetetraacetic acid), resuspended in 150 μL FACS buffer and analyzed using an Aurora spectral flow cytometer (Cytek Biosciences, Fremont, CA, USA). Antibodies used for assay validation were based on a marker set routinely used in our research setting. A large batch was used to circumvent having to use different batches during the validation process: SSEA4 (fluorescein isothiocyanate, 560126; BD), TRA-1-60 (BV510, 563188; BD), OCT3/4 (PerCP Cy5.5, 560794; BD), SOX2 (Alexa Fluor 647, 560294; BD) and NANOG (phycoerythrin, 560483; BD).

Results and Discussion

Design of validation protocols

Before onset of the validation studies, a validation protocol was generated for each individual analytical procedure described. As dictated by ICH Q2 [22], a validation protocol needs to contain pre-specified critical aspects to be tested and pre-defined acceptance criteria for pass or fail of a product to be tested with that particular test. The defined critical aspects and pre-set acceptance criteria are described in the following sections and depicted in Table 2.

REV analysis

Critical aspects for the detection of REVs were set as follows: (i) sensitivity of the assay allowing detection of residual vectors as specified for the assay's limit of detection, (ii) specificity of the primers in the context of gDNA to exclude non-specific primer annealing and (iii) assay reproducibility to ensure that different runs of the same sample provide the same result. In accordance with the guidelines provided by the Dutch Bureau for Genetically Modified Organisms under Genetic Modification Permit IM-MV 20-016, the detection limit of the assay was defined as the ability to detect one copy per 1000 cells. In addition, the passage number at which to start testing was assessed to ensure balanced product quality and production costs. The acceptance criteria formulated before assay validation were as follows: no qPCR signal detected at 35 passages = PASS, qPCR signal detected within 35 passages = FAIL.

Assessing functional pluripotency of hiPSCs

The critical aspects identified for the validation plan were (i) consistency of differentiation when using a commercial differentiation kit (insufficient cell coverage [cell confluency] on Matrigel-coated coverslips and poor differentiation may result in absence of lineage-specific markers), (ii) antibody specificity to differentiate between germ layers (non-selective binding of antibodies would lead to an unreliable assessment of the differentiation potential) and (iii) reproducibility (the assay must provide similar results when differentiated and analyzed multiple times). As scoring confocal images is prone to operator bias, assay interpretation was performed by multiple operators. The acceptance criteria formulated before assay validation were as follows: at least two of the three lineage-specific markers are present in each lineage = PASS, less than two of the three lineage-specific markers are present in at least one of the lineages = FAIL.

Assessing markers of the undifferentiated state

The critical aspects identified when setting up the validation plan were (i) antibody sensitivity and specificity, (ii) antibody panel validation testing appropriate gate setting using fluorescence minus one (FMO) controls and (iii) reproducibility of the assay. Acceptance criteria were set as follows: the expression of individual markers in at least 75% of hiPSCs and the expression of all markers in a single cell in at least 70% of hiPSCs = PASS, percentage less than 75% for

Table 2
Acceptance criteria set in the validation plan.

QC test	Outcome not accepted: FAIL	Accepted outcome: PASS
Residual episomal vector (REV)	A PCR signal obtained within 35 cycles on the *EBNA-1* target	No PCR signal obtained within 35 cycles on the *EBNA-1* target and a positive PCR signal for the control target HK2.
Markers of the undifferentiated state	<75% of cells positive for markers SSEA-4, Tra-1-60 and OCT3/4 and <70% of cells positive for all 3 markers	>75% of cells positive for markers SSEA-4, Tra-1-60 and OCT3/4 and >70% of cells positive for all 3 markers
Tri-germ layer differentiation	Less than 2 out of 3 markers positive for each of the 3 germ layers tested	At least 2 of 3 markers positive for each of the 3 germ layers tested
Action taken	Reject cell line for MCB generation	Accept cell line as possible candidate for MCB generation

individual markers and less than 70% for all markers = FAIL. Any lines with a PASS on all QC tests would be candidates to take forward for MCB production. Any lines with a FAIL would be rejected and discarded.

Assay validation for REV analysis

Assay sensitivity

To evaluate the assay's sensitivity, a dilution series (10^4, 10^3, 10^2 and 10 plasmid copies) was produced from all three reprogramming plasmids (pCXLE-hSK, pCXLE-hUL and pCXLE-hOCT3/4), and each series was tested in triplicate. As a target for REV analysis, we used primers annealing to part of the *EBNA-1* gene present on all three reprogramming vectors. A melting curve was produced to exclude the presence of primer dimer formation or non-specific primer annealing. The PCR assay consistently identified a minimum of 10 copies in all samples within 35 cycles for all three plasmids (Figure 1). To ensure that the agreed detection limit of the assay could be met, the minimum input for assay validation was set to 120 ng of gDNA equaling 20 000 cells to allow the identification of at least 20 copies equaling one copy per 1000 cells. The estimation of cell numbers was derived from the assumption that each human cell contains 6 pg of DNA [23]. It is worth highlighting that clear separation between pre- and post-processing workspaces is crucial to mitigate contamination when performing qPCR, especially when working with plasmid dilution series as input material.

Assay specificity

To test the primer sensitivity and specificity in the context of gDNA, an *EBNA-1*-positive control line (LU99_EBNA-1) was used, in which a single copy of part of the *EBNA-1* gene was inserted using STRAIGHT-IN [24] Single copy integration was confirmed using ddPCR (see supplementary Figure 1). The *EBNA-1* primers showed specificity in the context of gDNA. Using a dilution series (20 000, 2000, 200, 20 and two copies), we showed that 20 copies could consistently be detected in line with the detection limit observed using episomal vector DNA. The parental cell line (LU99), in which *EBNA-1* is not present, tested negative in the assay. The gDNA integrity was tested using control primers annealing to *HK2*. This control primer set was run in parallel in all assays to confirm gDNA input. Figure 2 shows the standard curve and Cq values for *EBNA-1* and *HK2* of a representative experiment.

Assay reproducibility

The reproducibility of the assay was tested by analyzing the same induced pluripotent stem cell (iPSC) lines in three different PCR runs using the exact same plate set-up. Six hiPSC lines, two hiPSC lines from three donors each, were used. The presence of REVs in each tested line was known prior to performing reproducibility testing. Lines (DDP08L4, DDP08L06, DGP08L03, DGP08L06, DHP08L04 and DHP08L10) were subjected to REV analysis in three separate PCR runs employing both the *EBNA-1* test primer set and the *HK2* control primer set. Results for the three PCR runs are summarized in Table 3. Although Cq values in the three runs varied slightly, the results of the analysis were consistent for all test runs.

Optimal time point to perform the assay

Screening for REVs for hiPSC line selection was a crucial in-process control step within our GMP-grade manufacturing process in which hiPSC lines for clinical use were manufactured. A pivotal objective was to conduct this screening at the optimal time point wherein the highest proportion of REV-negative lines could be identified while also minimizing passage numbers. Prolonged culture increases manufacturing costs and might affect genomic stability [25,26], underscoring the significance of early and effective screening for a cost-efficient and safe hiPSC product. In light of these considerations, we evaluated 18 GMP-generated hiPSC lines derived from four distinct donors at passages four, six, eight and 10. Passage one was defined as the moment after individual colony selection.

Our findings revealed that at passages four and six, over 50% of the examined hiPSC lines displayed detectable residual vectors (Figure 3). Screening at such early passages would therefore result in the exclusion of more than half of the generated hiPSC lines, potentially eliminating valuable candidates from the manufacturing pipeline. Consequently, this would compel the selection and maintenance of a greater number of clones post-transfection to enhance the likelihood of successfully establishing GMP-compliant hiPSC lines, ultimately increasing both labor demands and manufacturing costs. By contrast, we found that by passage eight, 72% of the GMP-compliant lines displayed undetectable levels of reprogramming vectors. Our findings advocate for the implementation of screening between passages eight and 10 as the optimal window. This approach enables the exclusion of lines with episomal vector retention while minimizing the rejection rate to 25%. This aligns with previous reports [27–31] and offers the dual benefit of ensuring data-driven decisions in the selection of hiPSC lines while circumventing unnecessary prolonged culture periods and ultimately helping to reduce the potential risk of adverse effects on genomic integrity.

One line of donor 4, D4L18, remained *EBNA-1*-positive in the qPCR assay. The Cq value remained constant until passage 10 and was similar to the positive control. Of the 18 tested lines, the qPCR data suggest that D4L18 is the only line with potential genomic integration of the *EBNA-1* sequence.

In addition to the tests described for assay validation, additional obligatory testing was required for our GMP hiPSC production process according to country-specific and local regulations. The Dutch Bureau for Genetically Modified Organisms required WGS with 50× sequence coverage to confirm the absence of integrated vectors or fragments thereof. Lines to be taken forward for MCB generation

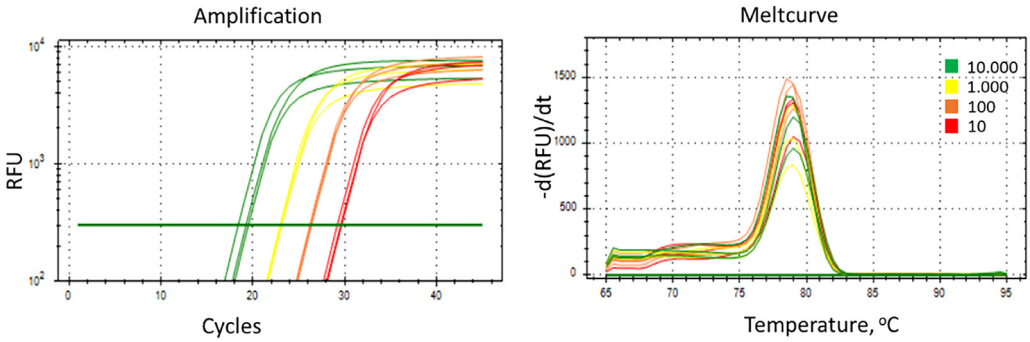


Fig. 1. Test of reprogramming vector dilution series displaying amplification and melting curve. −d(RFU)/dt, differential of relative fluorescence units over time; RFU, relative fluorescence units.

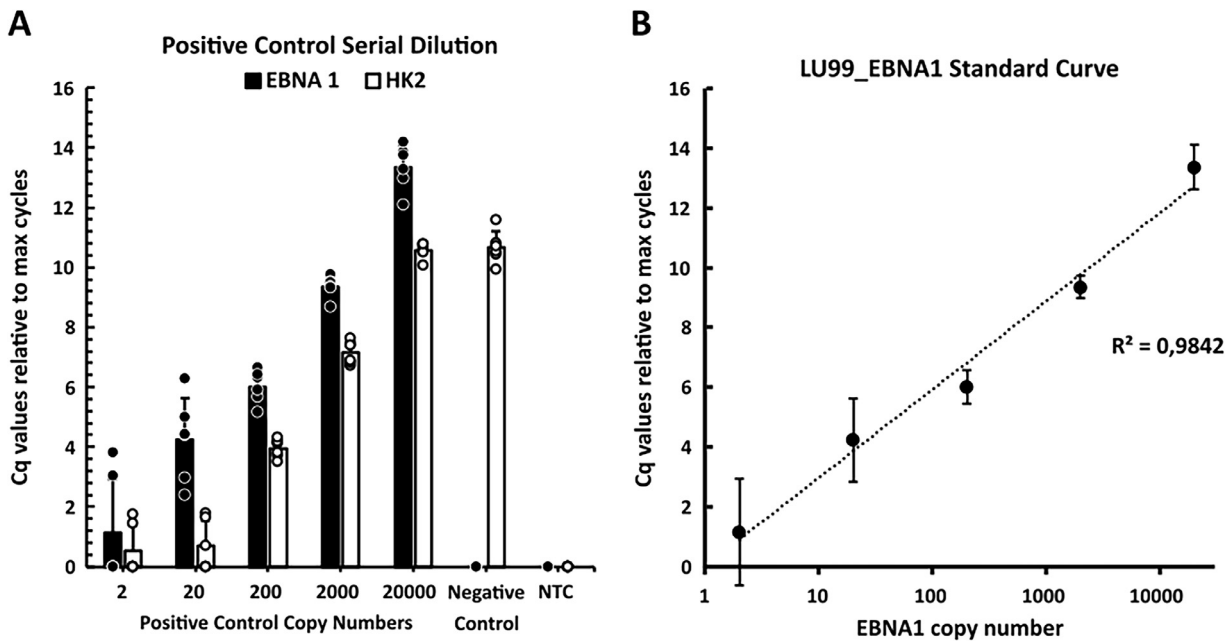


Fig. 2. REV specificity analysis with positive control dilution series, negative control and NTC (n = 6). (A) *EBNA-1* and *HK2* Cq values normalized to the max number of cycles (35). (B) *EBNA-1* standard curve constructed with serial 10-fold dilution of LU99_EBNA-1. Normalized Cq values of positive control plotted against the logarithm of known copy numbers. max, maximum; NTC, no template control.

(D2P10L1, D2P10L2, D2P10L3, D4P9L1, D4P9L10, D4P9L17) were subjected to WGS. None of these lines showed (partial) genomic integration of the reprogramming vectors [7]. Although line D4L18 was rejected based on the REV QC and would not have been taken forward for WGS analysis, we nevertheless tested this line. D4L18 indeed showed integration of the reprogramming vector (see supplementary Figure 2). The absence or presence of genomic integration of reprogramming vectors, as determined with both qPCR and WGS methods, highlights the validity of the chosen methodology for QC testing.

Dutch legislation on genetic modification considers episomal reprogramming a genetic modification procedure requiring donor testing for the presence of pathogens for which regulatory elements are present in the episomal vectors (e.g., EBNA-1 of EBV). It is important to note that epidemiological studies have shown that the infection rate of EBV in the population exceeds 95% [32]. Hence, testing of donor input material for GMP production processes is deemed critical. EBNA-1 is the sole protein expressed by EBV in proliferating cells across all forms of latent infections [33]. Sequences of *EBNA-1* have been utilized for the detection of EBV DNA in plasma and PBMCs for diagnostic purposes [34]. To mitigate any potential confounding effects of EBV infection on the REV analysis, gDNA was extracted from PBMCs of each donor and examined using the described *EBNA-1* primer set. For all tested PBMC samples, no signal was detected after 35 cycles (see supplementary Figure 3). In addition, local regulations required the produced hiPSCs of donors who were reported to be positive for EBV antibodies in serology testing (donors 3 and 4) to be further tested by LUMC's accredited routine medical microbiology testing laboratory. The qPCR assay used by the routine lab makes use of the *BNRF1* gene as the EBV target. Using this different target allows for the differentiation between EBV infection and reprogramming-introduced *EBNA-1* in the hiPSCs. The *BNRF1* gene is not part of the reprogramming vectors; hence, this PCR test detects only EBV infection. None of the hiPSC lines tested from donors 3 and 4 were positive in the routine assay, verifying the validity of our qPCR assay.

As with any analytical test, the qPCR method has limitations wherein a small percentage (<0.1% [i.e., limit of detection], less than 1:1000 cells) of vector-containing hiPSCs in the released batch cannot be detected. The advice is to perform REV analysis after generating an MCB and before final differentiation to a clinical product to exclude the possibility of clonal expansion of vector-containing hiPSCs. In addition, integration of partial plasmid sequences lacking *EBNA-1* will not be detected. Therefore, WGS on lines to be taken forward for MCB production is highly recommended.

Table 3
Reproducibility test of REV assay. Average *EBNA-1* data obtained in the three validation runs (n = 3). ND, not detectable.

Lines	Validation Run 1		Validation Run 2		Validation Run 3	
	Cq Value	Status	Cq Value	Status	Cq Value	Status
DDP08L04	34	Fail	34	Fail	32	Fail
DDP08L06	31	Fail	33	Fail	31	Fail
DGP08L03	ND	Pass	ND	Pass	ND	Pass
DGP08L06	28	Fail	27	Fail	28	Fail
DHP08L04	ND	Pass	ND	Pass	ND	Pass
DHP08L10	ND	Pass	ND	Pass	ND	Pass

Assay validation for assessing functional pluripotency of hiPSCs

Robustness of differentiation

Lineage differentiation is a stepwise process from pluripotency to lineage commitment. To validate the STEMdiff Trilineage Differentiation Kit, nine distinct hiPSC lines sourced from three different research donors were tested. The tested lines were as follows: donor D (lines two, four and seven), donor G (lines one, seven and 10) and donor H (lines four, five and 11). Subsequently, each line underwent evaluation against lineage-specific markers. The chosen marker set was already in routine use at LUMC's hiPSC hotel, providing consistent results, so we decided to validate this set of antibody conjugates. A large batch was used to circumvent the need for risk assessment, which might be

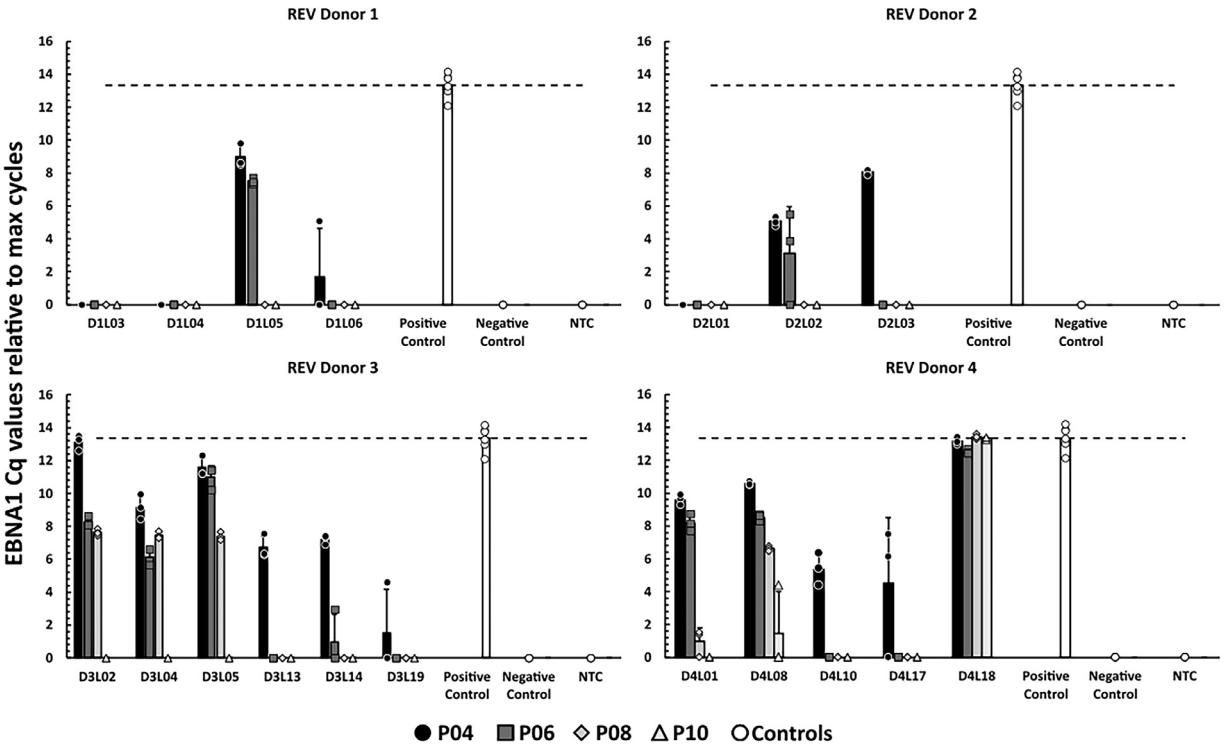


Fig. 3. REV qPCR. Screening of hiPSC lines from donors 1 to 4 at P04, P06, P08 and P10 for the presence of REVs. For lines in which episomal vectors were undetectable, the Cq value was set at the max number of cycles (35). The Cq values are plotted in relation to the max number of cycles (35) to visually represent the decrease in residual vectors throughout the passages. The four plots display the values for each hiPSC line (n = 3) and for the controls (n = 6). The dotted line represents the average Cq value of the positive control (one vector copy in one cell). max, maximum; NTC, no template control; P04, passage four; P06, passage six; P08, passage eight; P10, passage 10.

necessary when different lots are used. Detailed results are provided in Table 4, with corresponding confocal microscopy images illustrating the outcomes presented in Figure 4.

All tested cell lines exhibited adequate cell coverage on coverslips using the manufacturer's recommended cell seeding density, enabling staining and confocal analysis of a total of 27 coverslips. Of the nine tested lines, line 10 from donor G was scored as FAIL since in the mesoderm differentiation only vimentin was clearly detected. By contrast, Brachyury and CDX2 were deemed negative because of atypical staining patterns. The remaining eight lines demonstrated a positive signal for at least two of three markers across all germ layers. This aligns with the pre-determined PASS criterion in our QC testing. The results demonstrate successful differentiation across the three germ layers regardless of the donor source utilized and underscore the robustness of our procedure for assessment of functional pluripotency. It is important to note that line 10 displayed borderline cell confluency. In case of low cell confluency on coverslips, we advise refraining from further processing and analysis, as this might result in poor staining and unnecessary rejection of iPSC lines. Instead, differentiation should be repeated.

Antibody specificity

Antibody specificity and suitability of the antibody panels for this particular assay were tested by excluding staining of undifferentiated hiPSCs and background staining in non-corresponding germ layers. For this purpose, line DDP10L04 was used (Figure 5), and none of the antibodies recognized undifferentiated hiPSCs. As expected, three markers were detected in their respective lineages, with the exception of FABP7, which was undetectable in this particular hiPSC line. When using the antibody sets in non-corresponding germ layer differentiations, some staining was observed. In ectodermal cells, a signal for vimentin (mesoderm-specific marker) was detected in the Alexa Fluor 647 channel. This may suggest the presence of primitive neuroectoderm, as vimentin has been identified as a specific marker for astrocyte precursors [35]. In mesoderm-differentiated cells, a signal was detected with the Nestin antibody (ectoderm-specific

Table 4
Scores of lineage-specific markers. PASS = at least two of the three lineage-specific markers are present in each lineage. FAIL = less than two of the three lineage-specific markers are present in at least one of the lineages.

Donor	Endoderm			Mesoderm			Ectoderm			Status
	EOMES	FOXA2	GATA4	BRA	CDX2	VIM	NES	FABP7	PAX6	
D Line 02	+	+	+	+	+	+	+	+	+	Pass
D Line 04	+	+	+	+	+	+	+	-	+	Pass
D Line 07	+	+	+	+	+	+	+	-	+	Pass
G Line 01	+	+	+	+	+	+	+	-	+	Pass
G Line 07	+	+	+	+	+	+	+	-	+	Pass
G Line 10	+	+	+	-	-	+	+	-	+	Fail
H Line 04	+	+	+	+	+	+	+	+	+	Pass
H Line 05	+	+	+	+	+	+	+	+	+	Pass
H Line 11	-	+	+	+	+	+	+	+	+	Pass

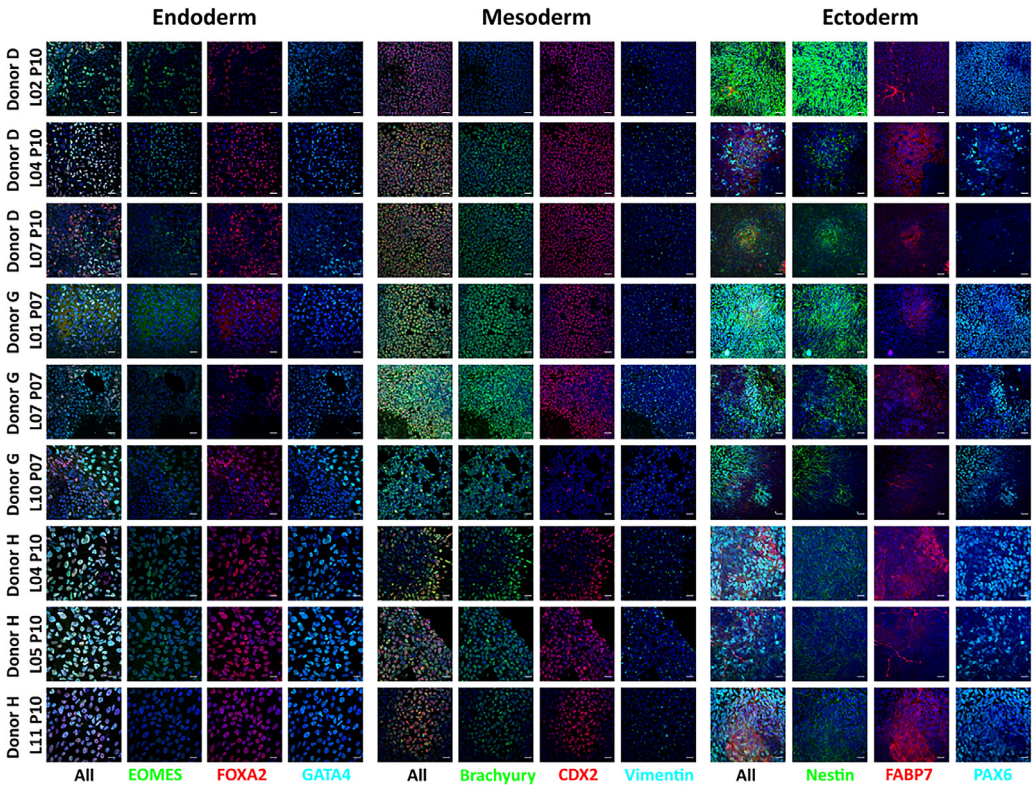
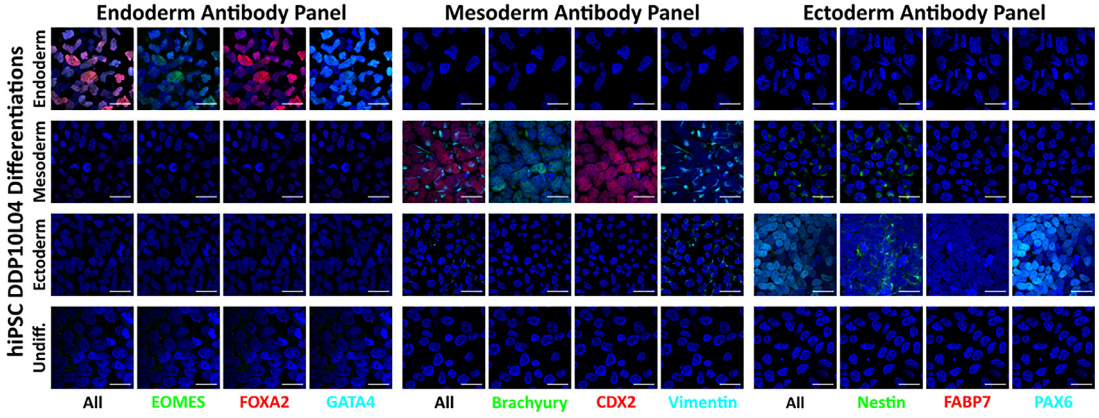


Fig. 4. Trilineage differentiation kit validation. Confocal microscopy of lines DDL02P10, DDL04P10, DDL07P10, DGL01P07, DGL07P07, DGL10P07, DHL04P10, DHL05P10 and DHL11P10 differentiated into endoderm, mesoderm and endoderm followed by immunofluorescence staining. DAPI is shown in blue. A representative image of one differentiation per germ layer and line is shown. Scale bar = 20 μm. DAPI, 4′,6-diamidino-2-phenylindole; L01, line one; L02, line two; L04, line four; L05, line five; L07, line seven; L10, line 10; L11, line 11; P07, passage seven; P10, passage 10.

marker) in the Alexa Fluor 488 channel. Although some cross-reactivity of antibodies with other lineages was observed, non-specific staining for non-corresponding lineages did not exceed one marker per lineage. Cross-reactivity for at least two of the three markers was not observed.

Assay reproducibility

To test the reproducibility of our assay, three individual operators performed staining and interpretation of confocal data independently. For this purpose, DDP10L04 cells were used. Each operator received one set of each germ layer to perform antibody



Staining Panel / Lineage	Endoderm			Mesoderm			Ectoderm		
	EOMES	FOXA2	GATA4	BRA	CDX2	VIM	Nestin	FABP7	PAX6
Endoderm	+	+	+	-	-	-	-	-	-
Mesoderm	-	-	-	+	+	+	+	-	-
Ectoderm	-	-	-	-	-	+	+	-	+
hiPSC (Undiff)	-	-	-	-	-	-	-	-	-

Fig. 5. Trilineage differentiation antibody specificity. Plus sign represents a positive staining result and minus sign indicates no staining detected. Boxes in green indicate the expected positive markers of each lineage and boxes in red indicate cross-reactivity of lineage-specific markers. Scale bar = 20 μm. Undiff, undifferentiated.

Table 5
Scores of lineage-specific markers for line DDP10L04 tested by three operators and analyzed under LSM 900 and SP8 confocal microscopes.

Operator (Confocal)	Endoderm			Mesoderm			Ectoderm			Pass/ Fail
	EOM	FOXA2	GATA4	BRA	CDX2	VIM	NES	FABP7	PAX6	
1 (Zeiss LSM900)	+	+	+	+	+	+	+	-	+	Pass
2 (Zeiss LSM900)	+	+	+	+	+	+	+	-	+	Pass
3 (Zeiss LSM900)	+	-	+	+	+	+	+	-	+	Pass
1 (Leica SP8)	+	+	+	+	+	+	+	-	+	Pass
2 (Leica SP8)	+	+	+	+	+	+	+	-	+	Pass
3 (Leica SP8)	+	+	+	+	+	+	+	-	+	Pass

staining and subsequent analysis using the LSM 900 Airyscan confocal microscope with ZEN software as well as the SP8 confocal microscope with LAS X software. The use of both microscopes aimed to assess comparability between the equipment. A summary of the results is presented in Table 5, with the corresponding confocal microscopy images illustrating the outcomes shown in Figure 6.

All three lineages consistently yielded positive scores independent of the operator or the imaging system. However, it is worth noting that the images were captured from different locations on the coverslip. The selection of the imaging area is operator-dependent, and as such minor discrepancies in the analyzed sets may arise, as exemplified by the FOXA2 staining by operator 3 in which images on the same coverslip were rated positive using the SP8 and negative using the LSM 900. Nevertheless, this variation did not alter the overall positive score for endoderm.

The trilineage differentiation test is time-consuming, and scoring of confocal images is prone to operator bias. There are various other methods to check for differentiation potential (e.g., using flow cytometry or based on molecular techniques). We nevertheless opted to use this test as a QC validation assay for our GMP production process, first because of its successful use in our research setting and second because of the recommendations by the International Society for Stem Cell Research, which state that unequivocal evidence of differentiation into progenitors of definitive endoderm, mesoderm and neuroectoderm should be based on multiple criteria. The tri-germ layer differentiation test allows for both morphological analysis and analysis of the expression of an appropriate combination of lineage-specific markers. When using the assay, one should be aware of the subjectivity of image-based analysis. We advise taking into account that for image analysis the selection of the area on the coverslip is of the utmost importance. Markers are not necessarily co-expressed in the same cell or present in the same area of the slide. Therefore, it is important to screen the complete coverslip systematically and capture images of different areas if required. This ensures a comprehensive evaluation of the totality of differentiated cells across the coverslip, reducing the potential of incorrectly scoring any of the lineage-specific markers. In addition, we advise having two independent operators score confocal images, as operator bias might be an issue for this part of the assay, and having the image analysis confirmed by an independent expert in the hiPSC field. Any line that fails to differentiate into one of the three germ layers would not be taken forward for MCB production, as it fails to meet the set selection criteria. GMP manufacture of hiPSCs is labor-intensive and production of an MCB is costly; therefore, correct selection of lines that have the potential to differentiate into multiple desired clinical products is preferred. Although the tri-germ layer assay gives an indication of the potential of the hiPSCs to differentiate into various clinical products, it does not guarantee that a specific product can be produced with a particular line. It is interesting to note that lines

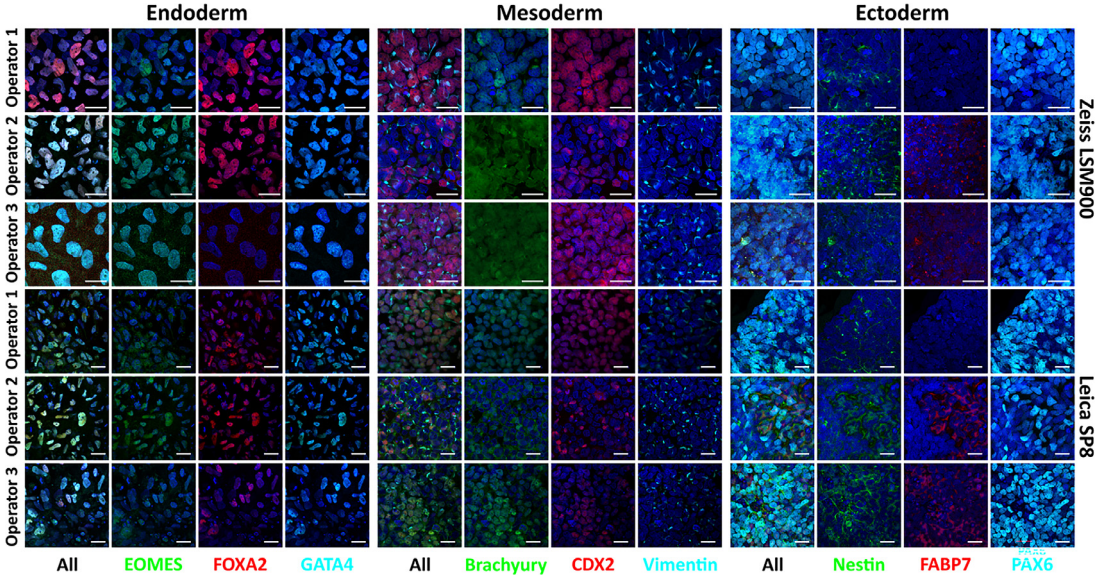


Fig. 6. Reproducibility validation of trilineage differentiation assay. Line DDP10L04 was differentiated, stained and analyzed by three independent operators and evaluated under two different confocal microscopes: LSM 900 Airyscan (×63) and SP8 (×40). Scale bar = 20 μm.

selected based on this QC assay and that were taken forward for MCB production were shown to be able to differentiate into all putative clinical products tested: pancreatic islets (endoderm); kidney organoids and cardiomyocytes (mesoderm); and keratinocytes, GABAergic interneurons and inner ear organoids (ectoderm) [7].

Assay validation for assessing markers of the undifferentiated state

Antibody specificity

Antibody specificity for distinguishing between undifferentiated and differentiated cells was assessed using HaCaT cells as a differentiated negative control in a 1:6 ratio with hiPSCs.

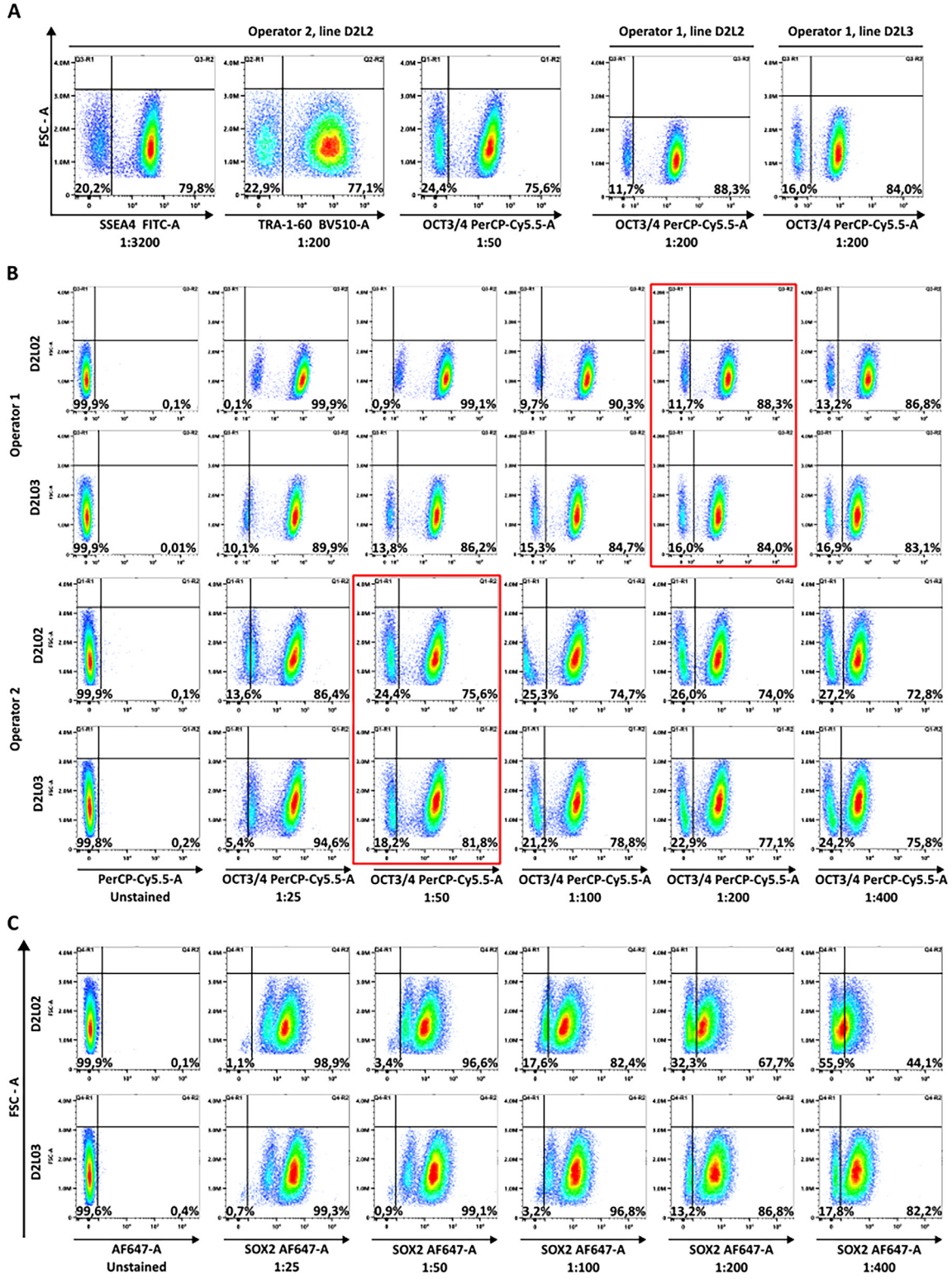


Fig. 7. Specificity and sensitivity of antibodies to markers of the undifferentiated state. (A) Dilutions selected for QC analysis. (B) Representative example of a dilution series showing OCT3/4. Red box indicates the dilution chosen by each operator. (C) No clear separation of HaCaT cells from hiPSCs using the SOX2 antibody. FITC-A, fluorescein isothiocyanate area; FSC-A, forward scatter area.

Recommendations for the use of markers assessing the undifferentiated state have been published [10,17] and are widely used in the iPSC research setting. This guided us in the choice of a marker set to initiate testing of antibodies against the following markers: OCT3/4, SOX2, NANOG, SSEA4 and TRA-1-60. A large batch of antibodies used successfully in our research setting was ordered to avoid the need for a risk assessment and possible comparability testing to exclude lot differences during validation testing. The final goal was to select a set of three markers comprising at least one surface marker and one intracellular marker. Antibody dilution series were tested to identify the optimal antibody concentration capable of separating positive and negative cell populations. The cutoff value for acceptable separation was set to less than 5% of the negative cell population in the positive gate. Selected antibody dilutions were based on the manufacturer's recommended dilution, including a 2 × 2-fold increase and decrease in dilution: SSEA4 (1:200, 1:400, 1:800, 1:1600 and 1:3200), TRA-1-60 (1:25, 1:50, 1:100, 1:200 and 1:400), OCT3/4 (1:25, 1:50, 1:100, 1:200 and 1:400), SOX2 (1:25, 1:50, 1:100, 1:200 and 1:400) and NANOG (1:12.5, 1:25, 1:50, 1:100 and 1:200). The titration experiments were executed by two independent operators using two GMP-generated lines: D2L02 and D2L03. Example FACS plots depicting the dilutions used for the QC assay are shown in Figure 7A. The results of a representative dilution series are depicted in Figure 7B. The optimal dilution for separating cell populations with OCT3/4 varied depending on the tested cell line. The individual preference of operators for the dilution to be used for OCT3/4 also differed. After repeated testing of different hiPSC lines with both OCT3/4 dilutions and obtaining cell line-specific results, we opted for the inclusion of two different concentrations in QC analyses to assess OCT3/4. Staining for SOX2 proved to be difficult (Figure 7C), with none of the antibody dilutions for SOX2 providing a result that matched the pre-set cutoff value of less than 5% negative cells in the positive gate. This antibody was therefore rejected for incorporation into the QC test. The optimal dilution of an alternative intracellular pluripotency marker, NANOG, was defined (data not shown). However, NANOG failed FMO (Figure 8) and was not included in QC testing.

Antibody–fluorophore panel screen

Multiple antibodies with different fluorophores in a single tube in a so-called multi-color FACS assay can lead to fluorescent spread. An FMO control visualizes whether a particular combination of fluorophores can be used in a single tube. To this end, all antibodies minus one are tested to check whether the signal of one fluorophore interferes with the interpretation of the next. To evaluate this phenomenon, the selected antibody dilutions were used to perform an FMO control experiment to ensure gates were properly set and adjusted for fluorescent spread. FMO was performed on three different hiPSC lines by two independent operators. The gate to distinguish between negative and positive cells was initially set on the unstained sample. The FMO cutoff value was set to a maximum of 10% spillover of all other fluorophores in the channel of the missing antibody. For SSEA4, TRA-1-60 and OCT3/4, the spillover of the negative population was less than 10%. The phycoerythrin-labeled antibody recognizing NANOG failed FMO and was therefore deemed not suitable for this multi-color flow cytometry panel and hence was not incorporated into the QC assay. Representative FACS results obtained are shown in Figure 8. It is important to note that a NANOG antibody with a different fluorophore that emits in a different spectrum might very well be

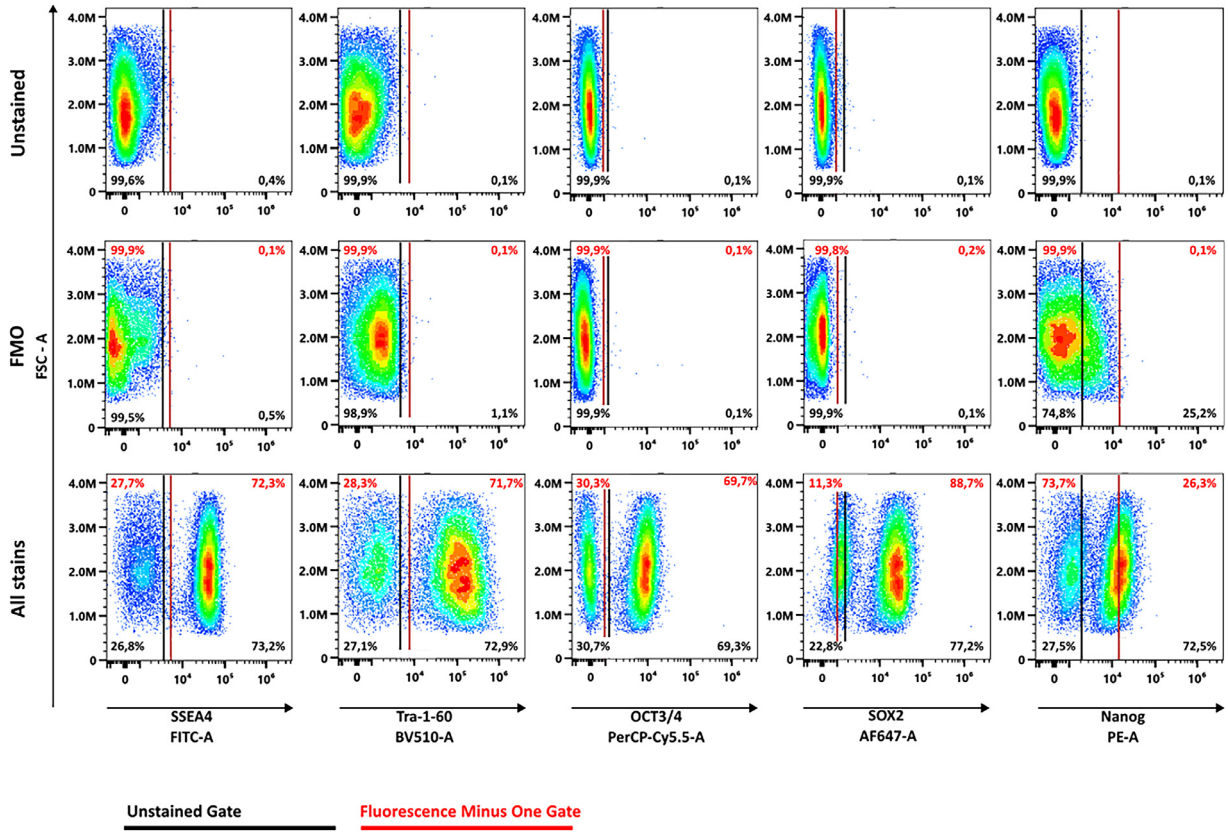


Fig. 8. Representative FMO experiment on hiPSC line D2L02 using SSEA4-FITC (1:3200), TRA-1-60-BV510 (1:200), OCT3/4-PerCP-Cy5.5 (1:200) and NANOG-PE-A (1:50). Percentages in black on the right and left side of the gate are set to the unstained control. Percentages in red on the right and left side of the gate are set to the FMO control. FITC-A, fluorescein isothiocyanate area; FSC-A, forward scatter area; PE-A, phycoerythrin area.

Table 6
FACS analysis performed by two operators. Percentage of positive cells for each stain and percentage of double positive cells for all antibody combinations.

Cell line	Operator	SSEA4	Tra-1-60	OCT3/4	Individual marker expression >75%
D2P16L01B	1	85,6%	92,4%	80,4%	PASS
	2	97,3%	91,8%	91,4%	PASS
D2P16L02	1	91,0%	91,3%	84,4%	PASS
	2	94,6%	86,8%	85,9%	PASS
D2P16L03	1	94,7%	94,7%	88,9%	PASS
	2	96,3%	94,6%	93,6%	PASS

Cell line	OCT3/4		SSEA4	Multiple markers expression 70%
	SSEA4	Tra-1-60	Tra-1-60	
D2P16L01B	91,6%	90,5%	92,0%	PASS
D2P16L02	86,0%	84,8%	87,0%	PASS
D2P16L03	93,6%	93,3%	94,8%	PASS

suitable. Our aim was to include three antibodies in the panel, and testing other fluorophore panels was not pursued.

Assay reproducibility

To assess the reproducibility of the assay, two independent operators performed independent tests on three lines: D2L01B, D2L02 and D2L03. The hiPSC lines were tested using the following multi-color antibody panel in a single vial: SSEA4-fluorescein isothiocyanate (1:3200), TRA-1-60-BV510 (1:200) and OCT3/4-PerCP-Cy5.5 (1:100). The results of both operators were scored (Table 6). Although differences in percentage between lines and between operators were observed, staining for SSEA4, TRA-1-60 and OCT3/4 produced consistent results. All three lines passed the set criteria (expression of individual markers in at least 75% of cells and simultaneous expression of multiple markers in at least 70% of cells).

Conclusions

Three hiPSC-specific GMP-compliant QC tests were successfully validated. Our iPSC-specific QC tests comprise a qPCR assay to check for REVs, which is needed when episomal vectors are used for reprogramming. Testing at passages eight to 10 was identified as the ideal time window to implement REV analysis. For pluripotency testing, a tri-germ layer differentiation assay was used. Although this is a laborious test, it allows assessment of hiPSCs to be based on multiple criteria (i.e., morphology and germ layer-specific marker expression). Markers of the undifferentiated state were assessed using two extracellular markers (SSEA4 and TRA-1-60) and one intracellular marker (OCT3/4). All QC tests passed the set acceptance criteria. These validated tests were integrated into our GMP-compliant hiPSC production process [7]. It is important to acknowledge that the methodology and materials used for our assay validation fall partly under patents of third parties and therefore might have licensing restrictions for use other than research.

Funding

This work received financial support from Starfish (grant no. LRMP-035).

Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

Author Contributions

Conception and design of the study: BW, PM and IW. Acquisition of data: JN, ES, NFN, LZ and ABA. Analysis and interpretation of data: BW, JN, PM, IW, CF, FC and RPD. Drafting or revising the manuscript: JN, BW and TR. All authors have approved the final article.

Acknowledgments

Plasmids pCXLE-hSK, pCXLE-hUL and pCXLE-hOCT3/4 were gifts from Shinya Yamanaka. Art work was designed and provided by Manon Zuurmond.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2024.04.004.

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